Int. Appl. No.

PCT/EP2005/050174

Int. Filing Date

:

January 17, 2005

AMENDMENTS TO THE SPECIFICATION

Please add the following header and paragraph on page 1, immediately after the Title of the Invention:

Related Applications

This application is a US National Phase of International Application No.: PCT/EP2005/050174, filed January 17, 2005, designating the US and published in English on August 25, 2005 as WO 2005/078075, which claims the benefit of European application No.: 04075430.1, filed February 11, 2004, the entire disclosure of which is hereby expressly incorporated by reference.

Please add the following header immediately before paragraph [0001] on page 1:

Field of the Invention

Please add the following header immediately before paragraph [0002] on page 1:

Background of the Invention

Please add the following header immediately before paragraph [0008] on page 3:

Summary of the Invention

Please replace paragraph [0014] on page 5 with the following amended paragraph:

The amino acid sequence corresponding to the amino acid sequence Thr195 to Leu199 in TEM-1 β-lactamase is located between the last two alpha helices of the all alpha domain. In class A β-lactamase these helices are helix 8 and helix 9. The alpha helices 8 and 9 are defined as sequences ARALATSLQAFA (SEQ ID NO: 42) and SEKRELLIDWMK (SEQ ID NO: 43) in BlaP and are defined as PAAMATTLRKLL (SEQ ID NO: 44) and LASRQQLIDWME (SEQ ID NO: 45) in TEM-1 β-lactamases, respectively, and those alpha helices which correspond to those in β-lactamases of the same class or of classes C and D.

Please replace paragraph [0017] on page 5 with the following amended paragraph:

The amino acid sequence corresponding to the amino acid sequence K239 to E245 of the AmpC β -lactamase is located between the last two alpha helices of the all alpha domain. In class C β -lactamase these helices, which correspond to helix 8 and helix 9 of class A β -lactamases, are defined as sequences IEDMARWVQSNL (SEQ ID NO: 46) and KTLQQGIQLA (SEQ ID NO:

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Please replace the Table 1 header on page 7 with the following amended header:

Table: 1 Comparison of corresponding helices 8 and 9 in β-lactamases. The numbering scheme for amino acids is according to ABL (see text). BlaP (SEQ ID NO: 5); TEM-1 (SEQ ID NO: 4); AmpC (SEQ ID NO: 38); BlaR-CTD (SEQ ID NO: 40).

Please add the following header and paragraphs immediately before paragraph [0027] on page 9:

Detailed Description of the Drawings

Figure 1 shows the 3D structure of TEM-1 β -lactamase. A: active site of the enzyme. B: highly tolerant position to exogenous polypeptide insertion.

Figure 2 shows the model of β -lactamase hydrolysis of penicillin substrate.

Figure 3 shows the sequence of restriction cassettes internalised in TEM-1 coding sequence. TEM-1 WT (SEQ ID NOs: 48 and 49); TEM-1 197 KpnI (SEQ ID NOs: 50 and 51); TEM-1 197 cartridge 1 (SEQ ID NOs: 52 and 53); TEM-1 197 cartridge 2 (SEQ ID NOs: 54 and 55).

Figure 4 shows the 3D structures of TEM-1 and BlaP β -lactamases. Arrows show the polypeptide insertion site.

Figure 5 shows the sequence of *SmaI* and *EcoRV* restriction site introduced in BlaP and BlaL coding sequence, respectively. BlaP Wt (SEQ ID NOs: 56 and 57); BlaP 211 SmaI (SEQ ID NOs: 58 and 59); BlaL WT (SEQ ID NOs: 60 and 61); BlaL 203 SmaI (SEQ ID NOs: 62 and 63).

Figure 6 shows the fold of the TEM-1 β -lactamase. The position of the permissive sites (filled square), the semi-permissive site (open square) and the non-permissive sites (grey square) are indicated.

Figure 7 shows the toxicity titration curve of the hybrid proteins.

Figure 8 shows the immunogenecity determined by ELISA for the TEM197H and TEM197STa. A) The presence of anti-TEM antibodies was estimated by coating 250 ng of TEM per well. B) The presence of anti-STa antibodies was estimated by coating 250 ng of GST-STa per well. The serum was diluted 100 fold in PBS buffer. The numbers below the columns of the diagram indicate different mouse individuals.

Figure 9 shows the titration curve of the anti-TEM IgG in the serum collected at day 56.

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Figure 10 shows the isotypic response against the carrier protein (TEM197H). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 11 shows the determination of the level of the anti-TEM IgG raised against TEM197H (1), TEM197STa (2), TEM216STa (3), TEM232STa (4) and TEM260STa (5). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 12 shows the construction of hybrid proteins of the TEM-1 β -lactamase wherein one or more repeated domains of the *Staphylococcus aureus* protein A (figure 12A) are internalised. A: protein A of *Staphylococcus aureus* is composed of five repeated domains indicated by letters E, D, A, B and C. These domains bind the antibody Fc region. S is the signal sequence. The sequence at the C-terminus is the peptidoglycan fixation domain (P). B: shows the structure of the E domain. Each of the repeated domains of protein A is organised into three α helices. C: the DNA coding for the repeated domains of protein A was amplified by PCR. D: the agarose gel is showing restriction analysis of different hybrid β-lactamase clones bearing 1, 2 or 3 domains of protein A. E: the SDS-PAGE gel analysis shows the hybrid β-lactamase proteins wherein one or three domains of protein A have been incorporated.

Figure 13 shows the titration curve of immobilised rabbit IgG by TEM-PA hybrid protein. The adsorbance is plotted against the amount of fixed rabbit IgG (ng).

Figure 14 shows the construction of the hybrid proteins of the TEM-1 β -lactamase where the B1 and/or B2 domain or domains of the *Streptococcus pyogenes* protein G were internalised. A shows that protein G is composed of 2 repeated domains, called B1 and B2 that bind to the antibody Fc region. They confer an affinity for the antibodies Fc region. S is the signal peptide sequence of protein G. B shows that each of the 2 domains is organised with a β -sheet and α -helices. C shows that the nucleotide sequence encoding for the repeated domains of the G protein were cloned into the TEM-1 β -lactamase sequence. D shows an SDS-PAGE of hybrid β -lactamase TEM-1 having 2 domains of protein G internalised.

Figure 15 shows the nucleotide sequence of insertion site of BlaP β -lactamase (SEQ ID NOs: 64 and 65) and BlaP-HA hybrid protein (SEQ ID NOs: 66 and 67).

Figure 16 shows a 12% SDS-PAGE gel electrophoresis of the BlaP and BlaP-HA β-lactamases after SFF partial purification of periplasmic fractions coming from *E. coli* strain

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transformed with pROGENO-1 BlaP(211/SmaI) and pROGENO-1 BlaP-HA. Transformed bacteria were grown over night on rich medium at 37°C.

Figure 17 shows a Western Blot analysis of the BlaP and BlaP-HA β-lactamases using monoclonal anti-HA antibody conjugated with peroxidase. Immunorecognised proteins were visualised by enhanced chemiluminescence detection.

Figure 18 shows the titration curve of immobilised rat IgG by BlaP-HA hybrid protein. The absorbance is plotted against the quantity of IgG1 of rat anti-HA in ng.

Figure 19 shows the agarose gel where PCR amplification products of TEM-1 (197/SmaI) and some hybrid TEM-1 hPLA₂ protein were loaded.

Figure 20 shows the primary structure of the hPLA2 (SEQ ID NO: 68) on which the various fragments internalised in TEM-1 are underlined (1, 2 and 3).

Figure 21 shows the Potentiometric measurement of a platinum electrode where rabbit antibodies were immobilised on functionalised aniline by succinimidyl group. Curve A: base line Pt/Pani/Pani-R/IgG/TemPA without substrate of the β-lactamase. Curve B: The release of protons starts with the addition of the substrate (benzylpenicillin) and the electrode potential increases proportionally with the quantity of substrate. Point 1, 2,6.10⁻⁴ M; point 2, 2,6.10⁻³ M; point 3, 2,6.10⁻² M; point 4, 2,6.10⁻¹ M; point 5, 5,2.10⁻¹ M.

Figure 22 shows the detection threshold between 5 and 100 ng of rabbit IgG binding to the Fc binding domain of *Staphylococcus aureus* protein A internalised into AmpC β -lactamase according to example 18.

Figure 23 shows binding of fluorescent β-lactam and antibody Fc-domain according to example 20. The BlaR-CTD_F514-PA of example 20 was acylated or not by fluorescent ampicillin and subsequently immobilized on a membrane. After saturation with non-fat dried milk 3%, Donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham Bioscience) were added. After washing and addition of ECL Immunodetection reagent (Amersham Bioscience) the slot-blot was revealed after 5 minutes. (A) represents non acylated and (B) represents acylated with Fluorescent ampicillin (B) BlaR-CTD F514-PA;

lane 1: 0, 0.3, 0.6, 0.9 μ g of total proteins;

lane 2: 1.2, 1.5, 1.8, 2.1 µg of total proteins;

lane 3: 2.4, 2.7, 3, 5 µg of total proteins.

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Please delete paragraphs [0078], [0079], and the text between these paragraphs on pages 23-26.

Please replace paragraph [00138] on pages 42-43 with the following amended paragraph:

In order to construct a new LPS-binding peptide, first of all two complementary primers corresponding to the LPS-binding amino acid sequence (Pro Ile Ile Lys Leu Leu Lys Leu Leu Lys Leu Leu Arg Arg Lys Leu Leu Lys Leu Leu Lys Leu Leu Pro Asp Gln Glu Phe Lys Gln) (SEQ ID Primer 5'-NO: 36) were hybridised. sequence: CCGATCATCAAACTTCTCAAGCTGCTTAAACTCCTGCGCCGGAAACTTCTCAAGCTG CTTAAACTCCTGCCGGATCAGGAGTTTAAGCAG-3' (SEQ ID NO: 19) $\tt CTGCTTAAACTCCTGATCCGGCAGGAGTTTAAGCAGCTTGAGAAGTTTCCGGCGCAG$ GAGTTTAAGCAGCTTGAGAAGTTTGATGATCGG-3' (SEQ ID NO: 20). Hybridisation is achieved by heat denaturation followed by a slow cooling stage. Double stranded oligonucleotide was inserted in the gene of the BlaP β-lactamase that was cloned beforehand in the expression vector pROGENO-1 and digested by Smal. After transformation, the bacteria were selected on LB agar plate + Spectinomycin (100 μg/ml final) and cephaloridin (50 μg/ml). At the end of the screening stages, BlaP β-lactamases were isolated where LPS-binding domain The affinity of the BlaP-LPS chimera proteins for LPS is now being was internalised. characterised. Hydrolysis tests on the chromogenic substrate nitrocefin reveal that the BlaP-LPS chimeras also retain β-lactamase activity.

Please add an abstract provided herewith as the last page of the Specification.